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A survey on the antimicrobial activity of *Lactobacillus* strains isolated from traditional dairy products in the historical district of Ilam, Iran

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Abstract

In recent years, the demand for the use of natural antimicrobial agents rather than chemicals as food preservatives is rapidly growing. The whole bacterial culture and neutralized supernatants of thirty-nine isolates were examined for antimicrobial activity by the spot-on lawn assay against ten indicator microorganisms. Based on the results, the cultures of all isolates showed antimicrobial activity. The maximum inhibition zone diameter was 38.2±0.1 mm. All the isolates had antimicrobial effects against *Bacillus cereus*, while 88.88% of the isolates did not show any inhibition zone around *Candida albicans*. Finally, eleven broad spectrum isolates selected; only isolate DL200 had antimicrobial activity even in a neutralized supernatant. The range of lactic acid production was 14.2-34.8 mg/g in cultured skim milk. There is a correlation coefficient between lactic acid production and the antimicrobial activity of isolates only against *Staphylococcus aureus* and *Bacillus subtilis*. However, there is no correlation between the lactic acid production of isolates and antimicrobial activity against other indicators, like *Salmonella typhimurium*, *Escherichia coli*, and *Pseudomonas aeruginosa*, and increasing lactic acid production did not increase antimicrobial activity. Selected isolates which use galactose as the sole carbon source for the production of CO2, were characterized as facultative heterofermentative and were identified as *Lactobacillus paracasei* with 99-100% identity based on the alignment results of their 16S rRNA gene. The use of selected isolates as preservatives in food not only inhibits the growth of pathogenic microorganisms but also prevents D-lactic acidosis in the host. Therefore, they are essential for children in the Autism spectrum who have high D-lactate in their systems.

1. Introduction

Microbial contamination in food products can cause food born disease or reduce the quality of food and usually occurs at different and even low temperatures, so the main challenge in the food industry is to remove microbial contamination by preservatives (Rai et al., 2016). Synthetic preservatives are extensively used to prevent the growth of microbial species. Scientific reports show that these chemical have serious impacts on human health with health consequences from chemical preservatives, like butylated hydroxyanisole, potassium bromate, propyl gallate, sodium nitrate, benzoates, being reported. They can trigger allergic reactions such as skin irritation,

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intestinal upset, and some breathing problems. Some of them have been linked to cancer in the kidneys and thyroid because they produce carcinogenic compounds when they are metabolized.

A healthy person's gastrointestinal microbials are balanced and beneficial microbes and pathogenic organisms can be found in coordination, but the presence of more pathogens than non-pathogens indicates bacterial infections (Guarner et al., 2003). Long-term consumption of food containing chemical preservatives reduces the number of beneficial microbes (Yang et al., 2014). Therefore, the use of natural antimicrobials without side effects to control spoilage microorganisms and foodborne pathogens is more in demand nowadays. Natural antimicrobial substances are obtained from plants or generally recognized as safe (GRAS) bacteria. The inherent immune system Lactobacillus produces antimicrobial substances such as organic acids, hydrogen peroxide, and bacteriocins, which destroy other related (narrow spectrum) or non-related (broad-spectrum) microbiota (Yang et al., 2014, Sumaira et al., 2010, Dinev et al., 2017). The antimicrobial effect of bacteriocin-producing *Lactobacillus* or their purified bacteriocins has attracted significant attention for improvement of the microbial safety of foods (Arokiyamary et al., 2012; Lucera et al., 2012).

The first step in obtaining the antimicrobial substances is the isolation of local bacteria from the environment or food. The isolation of *Lactobacillus* has always been the most potent means for obtaining useful strains (Patrick et al., 2014). Accordingly, many authors have conducted research isolating and investigating the characteristics of *Lactobacillus* from different types of foods from different geographical areas (Franciosi et al., 2009), for example *Lactobacillus* has been isolated from fermented milk in India (Lavanya et al., 2011), regional yogurts in Bangladesh (Hoque et al., 2010), traditional sourdough (Minervini et al., 2012), maize sourdough (Agati et al., 1998), cassava sourdough (Miambi et al., 2003), and fermented fish and rice food in Japan (Olympia et al., 1992). The author isolated *Lactobacillus* from traditional dairy products from the Ilam province, Iran (Ghobadi Dana et al., 2010).

The second step in obtaining useful strains is to screen *Lactobacillus* isolates for desired properties such as antimicrobial activity and production of useful materials. The author had screened *Lactobacillus* isolates for folic acid (Ghobadi Dana et al., 2010), acetaldehyde (Ghobadi Dana et al., 2011), and Lactic acid production (Soleimanifar et al., 2015).

The aims of this study were to screen the isolated *Lactobacillus* for antimicrobial activity, screen the isolates for bacteriocin production, and finally characterize the selected *Lactobacillus* isolates. The indicators were the three gram-negative pathogens, *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853; and four aerobic gram-positive bacteria, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Bacillus cereus* ATCC 10876, and *Bacillus subtilis* ATCC 6633; and one anaerobic gram-positive pathogen, *Clostridium perfringens* ATCC 13124; and two fungi, *Aspergillus niger* ATCC 16404 and *Candida albicans* ATCC 10231.

2.Materials and methods

2.1. Bacterial isolates and growth media

Lactobacillus strains were isolated from traditional dairy products samples prepared from the villages around Ilam province (N33°

36' 42.74", E 46° 25' 51.35") such as Dareshahr (N33° 2' 42.00", E 47° 18' 26.67"), Abdanan (N32° 59' 39.00", E 47° 22' 1.99"), and Sarabele (N33° 49' 49.18", E 46° 28' 19.66"), Iran. Chemicals were analytical grade and purchased from Merck or Aldrich (Germany). All the indicator strains for antimicrobial investigations were procured from the American Type Culture Collection (ATCC).

2.2. Preparation of isolated strains

After defrosting the storage cultures, they were revitalized in MRS broth (Merck, Germany) at 37°C for 16 h in anaerobic conditions. The optical density of the entire bacterial culture was 1.50±0.1 at 620 nm in a spectrophotometer (approximately equal to (1-2) ×108 CFU/ml). The cultures were centrifuged at 6000 g, 10 min. Moreover, in order to abolish the influence of organic acids and low pH, the supernatants were neutralized with 1M NaOH.

2.3. Preparation of indicator strains

The indicators such as *Salmonella typhimurium* (ATCC14028), *Escherichia coli* (ATCC25922), *Pseudomonas aeruginsa* (ATCC 27873), *Staphylococcus epidermidis* (ATCC 12228), *Bacillus cereus* (ATCC 10876), *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 25923) were cultured in nutrient broth and incubated at 37°C for 18 h in aerobic conditions. *Aspergillus niger* (ATCC 16404) and *Candida albicans* (ATCC 10231) were cultured in sabouraud dextrose agar and incubated at 25°C for 72 h in aerobic conditions. *Clostridium perfringens* (ATCC 13124) was cultured in a nutrient broth and incubated at 37°C for 18 h in anaerobic conditions. The optical density (OD) of the incubated indicators was 1.50±0.1 at 620 nm, which is approximately equal to (1-2) $\times 10^8$ CFU/ml bacteria and (1-5) $\times 10^6$ CFU/ml for fungi.

2.4. General characteristic of isolates

Bacterial colonies that were developed on MRS agar plates were picked and tested for catalase by placing a drop of 3% hydrogen peroxide solution on the cells. Then, the isolates were tested for coagulation, motility, nitrate reduction, indole production, forming spore, esculin and gelatin degradation, and gram staining according to the literature.

2.5. Detection of antimicrobial activity

The isolate cultures and neutralized supernatants of thirty-nine isolates were examined for antimicrobial activity by the spot-on lawn assay against ten indicator microorganisms. (Tendencia et al. 2004, Harris et al. 1999). *L. rhamnosus* (ATCC 9595), *L. acidophilus* (ATCC 314), *L. lactis* (ATCC 7830) were used as reference strains. MRS agar plates were spotted with separately 20 µl of the whole bacterial culture and neutralized supernatants of isolates and incubated overnight at 37°C under anaerobic conditions. Then 20 µl of the cultures of each indicator (1×10^8 CFU mL-1) were added to 7 ml agar-agar 0.7% and poured onto the cultured plates. After complete solidification, the plates were incubated for an additional 24 h at 37°C under aerobic conditions (Mohankumar et al., 2011). After incubation, the inhibition zones diameter was measured and considered its antimicrobial activity.

2.6. Growth in different substrates

The potential to grow in the different substrates and fermenting carbohydrates including cellobiose, fructose, galactose, glucose, gluconate, lactose, sorbitol, maltose, mannitol, mannose, melibiose, melezitose, sucrose, raffinose, trehalose, xylose, ribose,

arabinose, and rhamnose has been previously investigated (Kandler, et al., 1986). A volume of the basal medium containing bromocresol purple (0.16 g/L) was mixed with water to 1 L while maintaining a pH in the range 5.7± 0.2. The Basal medium (MRS ingredient) without glucose was dispensed in 3.0 ml and supplied with Durham tubes then sterilized at 121 °C for 20 min. Each sterilized carbohydrate (at 121 °C for 20 min) was added as the sole carbon source to the Basal medium in sterile conditions. Isolates were inoculated to the tubes containing liquid media supplied with Durham tubes. The culture was incubated at 37 °C for 48 h in anaerobic conditions.

2.7. Statistical analysis

All experiments were carried out in triplicate and an ANOVA followed by Student's test was conducted for analysis. A P-value≤0.05 was considered statistically significant and followed by Duncan's multiple range tests. Statistical significance was set at a confidence level of 95%.

2.8. Molecular identification

The genomic DNA from selected isolates was extracted by the CTAB method (Minas et al., 2011). DNA content was measured by means of nanodrop. The genomic DNA was used as a template (10ngµl-1, 1µl) for the identification at genus and species level, respectively. The amplification was carried out using Taq DNA polymerase, Qiagen (5U, 0.2 µl), dNTP 10mM (0.3 µl), buffer MgCl2 10X (2 µl), water (15.5 µl), and Lactobacillus genus-specific primers (10pmol, 0.5µl) (R16-1:5'-CTT GTA CAC ACC GCC CGT CA-3') and (LbLMA1: 5'-CTC AAA ACT AAA CAA AGT TTC-3') were carried out (PCR product=250bp) (Kwon et al., 2004). PCR amplification was conducted using the thermal program as 94ºC for 5 min, 30 cycles consisting of 94ºC for 40sec, 53°C for 40sec, 72ºC for 40sec, and a final extension step of 72ºC for 5 min in a thermal cycler (Eppendorf, Germany). The PCR products were visualized by 1% (w/v) agarose gel (Sigma-Aldrich) electrophoresis at 120 v, and amplified bands were viewed on a gel documentation system (Bio-Rad GDS, California, USA).

For identification of isolates at the species level, genomic DNA was used as the template DNA (10 ngµl-1, 1 µl) to amplify 16srRNA using Taq DNA polymerase, Qiagen (5U, 0.2 µl), dNTP 10 mM (0.3 µl), buffer MgCl2 10X (2 µl), water (15.5 µl), and specific primers (10 pmol, 0.5µl) including idl16r08F (5'-AGA GTT TGA TCC TGG CTC A-3') and idl16r09R (5'-TAC CTT GTT ACG ACT TCA CC-3') (Brosius et al., 1981). PCR amplification was performed using the thermal program as 94ºC for 5 min, 30 cycles consisting of 94ºC for 40sec, 57.5°C for 40sec, 72ºC for 1.5 min, and a final extension step of 72ºC for 7 min in a thermal cycler (Eppendorf, Germany). The PCR products were visualized by 1% (w/v) agarose gel (Sigma-Aldrich) electrophoresis at 120 v, and amplified bands were viewed on a gel documentation system (Bio-Rad GDS, California, USA). Bidirectional sequencing of purified, diluted PCR products were performed using the Sanger method with the ABI system by Macrogene, Korea. The sequences of PCR products were recorded in The National Center for Biotechnology Information, and the nucleotide sequences were compared with the sequences in the database by Basic Local Alignment Search Tool.

2.9. Construction of phylogenetic tree

A neighbor-joining algorithm (Saitou and Nei, 1987) was applied to generate the initial tree. The evolutionary distances was computed using the p-distance method (Nei and Kumar, 2000). The Minimum evolution (Rzhetsky et

al., 1992) tree was based on the16S rDNA sequence of *Lactobacillus* isolate. The bootstrap consensus tree inferred from 100 replicates (Felsenstein et al, 1985). Finally, MEGA 7.0 (Kumar et al. 2016) was used to visualize the phylogram trees and clusters (Tamura et al., 2007) (figure 1).

3. Results and discussion

3.1. Detection of antimicrobial activity

Based on the results, the whole bacterial cultures of all isolates showed antimicrobial activity and the diameters of the inhibition zone were 2.00±0.2 and 38.2±0.1 mm; only one isolate DL200 showed antimicrobial activity through its neutralized supernatant of isolate. A significant difference (p-value <0.05) was observed between the antimicrobial activity of the whole bacterial culture of isolates and the control strains. Table 1 shows the percentage of isolates, which were created different inhibition zone around the indicators.The antimicrobial activity of *L. rhamnosus* (ATCC 9595) and *L. lactis* (ATCC 7830) against ten indicators were less than 5.00 mm, and the antimicrobial activity of *L. acidophilus* (ATCC 314) against ten indicators were less than10.00 mm. The highest inhibition zone was observed around *Bacillus subtilis* (38.2±0.1 mm) and *Pseudomonas aeruginosa* (33.0±0.2 mm). All of the isolates showed antimicrobial activity against *Bacillus cereus*, 88.30% of the isolates exhibited antimicrobial effects against *Escherichia coli*, and 90.91% of the isolates had antimicrobial activity against *Salmonella typhimurium*. A significant difference (p-value ≤0.05) was observed between antimicrobial activity isolates against *Aspergillus niger* and *Candida albicans*. *Aspergillus niger* was inhibited by 98% of the isolates, while most of isolates did not demonstrate any inhibition zone around *Candida albicans.* This may be because the antimicrobial activities of the isolates were due to just organic acid production. Since the optimum pH for *Candida albicans* growth is 3-5 (Staib et al., 1969), it can survive in an acidic environment. Only one isolate (DL200), which has an inhibitory effect on the indicators even in the neutralized state, demonstrated an inhibition zone around *Candida albicans*.

Table 1: The percentage of isolates made different diameters (mm) of the inhibition zone around the indicators

Indicator strains	0mm	1-5mm	6-10mm	11-20mm	21-30mm	31-40mm
Salmonella typhimurium ATCC14028 n=33	3(9.09%)	24(72.72%)	4(12.12%)	1(3.03%)	1(3.03%)	
Escherishia coli ATCC25922 n=36	3 (11.70%)	16(41.02%)	15(38.46%)	4(10.26%)	1(2.56%)	
Psedomonas aeroginoza ATCC27873 n=36	1(2.77%)	12(33.33%)	9(25%)	7(25.20%)	4(11.11%)	1(2.77%)
Staphylococcus aureus ATCC25923 n=35	9(25.71%)	9(25.71%)	5(14.28%)	12(34.28%)	---	
Staphylococcus epidermidis ATCC12228 n=34	3 (8.82%)	20(58.82%)	7 (23.80%)	1(2.94%)	3(8.82%)	
Bacillus subtilis ATCC6633 n=34	1(2.94%)	9(26.47%)	9(26.47%)	6(17.65%)	7(20.59%)	2(5.88%)
Bacillus cereus ATCC10876 n=34	0(0.00%)	7(20.59%)	9(26.47%)	12(35.29%)	6(17.65%)	
Clostridium perferangenes ATCC13124 n=33	2(6.06%)	13(39.39%)	8(24.24%)	6(18.18%)	4(12.12%)	
Aspergillus niger ATCC16404 n=35	4(1.43%)	11(31.43%)	6(17.14%)	3(8.57%)	11(31.43%)	
Candida albicans ATCC10231 n=36	32(88.88%)	3(8.33%)	1(2.77%)	0(0.00%)		

On the other hand, all the isolates had antimicrobial effects on *Bacillus cereus*. Since the optimum pH for *Bacillus cereus* growth is in the range 4.9–9.3 (Sutherland et al., 1996), it is very sensitive to acidic conditions and cannot survive at this pH level. It seems that the

antimicrobial effect of the isolates is related to the production of acid and the creation of an acidic environment, therefore, they have a strong effect on *Bacillus cereus* and no effect on *Candida albicans.*

The results of antimicrobial activity based on the inhibitory diameter zone in mm for the isolates against indicators showed eleven isolates had higher antimicrobial activity in comparison to others as illustrated in Table 2. A significant difference (p-value <0.05) was observed in the antimicrobial activity of LD140 and other isolates against *Bacillus cereus* (25.50±1.50 mm and DL131b and other isolates against *Pseudomonas aeruginsa* (34.66±2.51 mm). A significant difference (p-value ≤0.05) was also observed between the antimicrobial activity of DL200 and other isolates against *Salmonella typhimurium* (24.00±1.71 mm), Escherichia coli (26.66±1.71 mm), Bacillus subtilis (35.13±1.02 mm), and Clostridium perfringens (25.00±1 mm). DL200 was the only isolate which showed antimicrobial activity on microbes after neutralization of supernatant. Our results agreed with the findings of Kaushik et al. which observed antimicrobial effects on *Bacillus cereus* of about 29 mm, but the *Bacillus cereus* in this report showed more antimicrobial effects compared to the results of Kraiyot et al. (2017).

The antimicrobial effects against *Escherichia coli* reported by Kaushik et al. (2009), Anas et al. (2008), and Kraiyot et al. (2017) were 16-20 mm, 16 mm, and 0.1-10 mm, respectively. The results of this study showed that the maximum antimicrobial activity against *Escherichia coli* was 28 mm, which is more than the results of Kaushik et al. (2009) and Kraiyot et al. (2017). Kaushik et al. (2009) and Kraiyot et al. (2017) also reported that the antimicrobial effects against *Staphylococcus aureus* was 14 mm and 15 mm, respectively; whereas, in this study the maximum antimicrobial effect against *Staphylococcus aureus* was considerably higher at 21-30 mm. The results of antimicrobial effects against *Salmonella typhimurium* agree with the outcomes of Anas et al. (2008) at 28 mm and were higher than the findings of Kaushik et al. (2009) at 20 mm.

Table 2: Evaluation the antimicrobial activity of selected and control strains against ten indicators

Indicators	Inhibition zone diameter(mm)													
	1	2	3	4	5	6	7	8	9	10	11	*b*	*c*	*a*
S.typhi	+	+	+	++	+	+	+	++	+++	++++	+	+	-	+
E. coli	+	+	-	+	++	+	-	++	-	++++	+	+	+	-
P.aeruginsa	+++++	++	++	+++	++	++	++	+++	++	-	+++++	+	-	-
S.epidermidis	++	+	+	++	+	++	+	++	++++	++	++	++	+	++
S.aureus	+++	++	-	+++	++	+++	++	+++	+++	++	+++	+	-	-
B.cereus	+++	+++	++	+++	+++	+++	+++	++++	+++	+++	+++	++	+	+
B.subtilis)	++++	++	+	++++	+++	+++	+++	++++	++++	+++++	+++	+	+	+
Cl.perfringens	+++	++	+	+++	++	+++	++	+++	++++	++++	+++	+	+	+
A.niger	-	++	++++	++++	++	++++	++++	++++	+	++++	++++	+	+	+
C.albicans	-	-	-	-	-	-	-	-	-	+	-	-	-	-

a :L. rhamnosus (ATCC 9595), b :*L. acidophilus*(ATCC 314), c :*L. lactis*(ATCC 7830),1:DL131b,2: DL99, 3:DL108, 4:DL132,5:DL104, 6:DL147, 7:DL130. 8:DL140, 9:DL124,10: DL200, 11:DL148 - = No inhibition zone, + = Radius of clear zone 0.1–5.0 mm, ++ = Radius of clear zone 5.0–10.0 mm, +++ = Radius of clear zone 10.0 -20.0mm ++++= Radius of clear zone20.0 -30.0mm, +++++= Radius of clear zone more than 30.0mm

According to Soleimanifar et al.'s report, the lactic acid production of these isolates varied considerably from14.2-34.8 mg/g, so that 87.34% of the isolates produced 15.00-25.99

mg/g lactic acid. Table 3 illustrates the percentages of isolates which produced lactic acid in different amounts.In Table 4 shows the lactic acid production of the isolates. A significant difference was observed between the lactic acid production of DL132 (34.83±3.25 mm) and the other isolates (p-value ≤0.05), but this relation was not observed between the antimicrobial activity of DL132 and the other isolates.

Table 3: Percentage of isolates that produced lactic acid in different amounts

Lactic acid production mg/g	15.00>	15.00-19.99	20.00-25.99	26.00-29.99	30.00-35.00
Percentage of isolate	7.60%	65.82%	21.52%	3.80%	1.26%

Isolate DL200, which produces relatively little lactic acid, showed substantial antimicrobial activity compared to the other isolates. It seems that its antimicrobial properties are due to the production of substances like bacteriocins. The Pearson correlation coefficient between lactic acid production and the antimicrobial activity of isolates against *Staphylococcus aureus* and *Bacillus subtilis* was significant with a positive relationship between the variables. The results demonstrate that when lactic acid production grows, the antimicrobial activity of isolates against *Staphylococcus aureus* and *Bacillus subtilis* rises (p-value <0.05).

T The results reveal that the antimicrobial effect stems from not only the presence of lactic acid but also other byproduct of fermentation. But, no correlation existed between lactic acid production and the antimicrobial activity of isolates against *Candida albicans, Aspergillus niger, Salmonella typhimurium, Staphylococcus epidermidis, Bacillus cereus, Pseudomonas aeruginsa, Clostridium perfringens,* and *Escherichia coli* (p-value ≥0.05).

Table 4. Lactic acid production of isolates (mg/g)

Isolates	Lactic acid production mg/g
DL200	16.50±0.70
DL131b	15.50±0.96
DL132	34.83±3.25
DL140	17.30±0.34
DL130	14.20±0.60
DL104	30.33±0.37
DL108	31.23±1.42
DL99	21.13±0.70
DL124	20.96±0.50
DL147	29.46±1.11
DL148	25.46±0.90

3.2. General characteristic

Isolates were gram-positive, rod-shaped Lactobacilli, catalase-negative, nitrate-negative, indole-negative, not spore-forming, non-motile, esculin-negative and gelatin-negative with the ability to grow in coagulate milk at 45°C.

3.3. Growth in different substrate

The results of the ability to survive in different substrates revealed that 100% of the Lactobacillus isolates grew well and produced acid when the sole carbon source was glucose, lactose, galactose or ribose. The predominant isolates could ferment these carbon sources and produce CO2, hence the isolates were characterized as a heterofermentative lactic acid producer. (Haywarda et al., 1957, Fatih Ortakci, 2015). The heterofermentative *Lactobacillus* not only ferments pentoses (ribose), but also they can use hexoses to produce lactic acid and other secondary products including acetate, ethanol, CO2, formate, succinate, diacetyl, acetoin and acetaldehyde (Salvetti, 2014). This confirms the lack of correlation between the production of lactic acid and the antimicrobial effect of the isolates. The antimicrobial effect is, therefore, a result of other by-products of hexose fermentation, except lactic acid which can

display antimicrobial effects (Medina, 2011).More than 50% of the isolates could grow and produce acid on fructose (hexose monosaccharides), mannose, trehalose, cellobiose, maltose and sucrose (disaccharides) and also in mannitol, salicin, and sorbitol (alcoholic sugar) by using them as a substrate. Thus, the population of isolates prefer disaccharides (except melibiose) and alcoholic sugar compare to other substrates. It seems that breaking the alpha (1-6) bond is difficult for this population of isolates. Less than 5% of the isolates could produce acid by using melezitose as a carbon source. Table 5 shows the acid-producing ability percentage of the isolates. The results indicate that the percentage of isolates which can produce acid from lactose, galactose, trehalose, and mannitol is greater than the *Lactobacillus* reported by Melinda et al. (2005). The proportion of isolates that can produce acid from glucose is similar to *Lactobacillus crispatus*, *Lactobacillus jensenii,* and *Lactobacillus gasseri* reported by Melinda et al. (2005).Sugar fermentation of the selected isolates were showed in table 6.

Table 5. The percentage of acid producing ability and sugar fermentation of the isolates

Carbohydrate	Pentose mono saccharides			Hexose mono saccharides			Disaccharides							Trisaccharides		Alcoholic β-glucoside			Others				
	ARB	XYL	RIB	GLU	GAL	FRU	LAC	TRE	MNE	MAL	CEL	SUC	MEL	RAF	MLZ	MAN	SAL	SOR	INO	RHM	ESC	AMD	GLN
Lactobacillus species	36	36	100	100	90	82	100	100	100	90	90	82	18	18	0	100	100	82	18	27	0	18	0

a the tests with a positive result were included: ARB: arbinose; GAL:D-galactose; GLU:D-glucose; FRU:D-fructose; MNE:D-mannose; MAN:D-mannitol; INO: Inositol; RHM: rhamnose; RIB: ribose; ESC: esculin; SAL: salicin; CEL:D-cellobiose; MAL:D-maltose; LAC:D-lactose; MEL: melibiose; MLZ: melezitose; TRE:D-trehalose; RAF:D-raffinose; AMY: amygdalin; SOR: sorbitol; SUC:D-sucrose; XYL: xylose ; GLN: gluconate, gas: gas production.

Table 6. Sugar fermentation of the selected isolates

Carbohydrate	**Pentose mono saccharides**			**Hexose mono saccharides**			**Disaccharides**							**Trisaccharides**		**Alcoholic β-glucoside**			**Others**				
Lactobacillus species	ARB	XYL	RIB	GLU	GAL	FRU	LAC	TRE	MNE	MAL	CEL	SUC	MEL	RAF	MLZ	MAN	SAL	SOR	INO	RHM	ESC	AMD	GLN
DL200	+	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	+	-	-	-
DL131b	-	-	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	-	-	-	-
DL132	+	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	-	-	-
DL140	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	-	-
DL130	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	+	+	-	-	-	+	-
DL104	-	-	+	-	-	+	+	+	+	+	+	+	-	+	-	+	+	+	-	-	-	-	-
DL108	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	-	-	-	-	-
DL99	+	-	+	+	+	+	+	+	+	+	-	+	-	-	-	+	+	+	-	-	-	+	-
DL124	-	+	+	-	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-
DL147	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-
DL148	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-	-	-	-

3.4. Molecular identification

All eleven selected isolates were identified as *Lactobacillus* on the genus level. Among them, DL132 displayed a high potential for lactic acid production and DL200, LD140, and LD131b showed high antimicrobial properties and were

identified as *Lactobacillus paracasei*. The results of sequencing the 16S rRNA gene sequence of the isolates were reported in NCBI. The results of molecular identification of five selected isolates is given in Table 7. The results of the Mega blast demonstrates that 16S rRNA sequences of the isolates had 99%-100% identity with *Lactobacillus paracasei*. The results of the biochemical identification of selected isolates were confirmed by the molecular identification. Hence, the selected isolates belong to the *Lactobacillus casei* group, are facultative heterofermentative and can form L (+)-lactic acid and other organic acids (Salvetti, 2012).

3.5. Construction of phylogenetic tree

The analysis involved seven nucleotide sequences, and there were a total of 928 positions in the final dataset. The evolutionary history indicated branches corresponding to partitions reproduced in less than 5% cut off. That is, fewer than 40% alignment gaps, missing data, and ambiguous bases were allowed at any position. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches (Felsenstein et al., 1985).

Table 7. Result of molecular identification of five selected isolates

Isolates	Number of nucleotides	%Identity	Nearest type strains	PTCC	Accession number
DL200	1,392 bp	100%	*Lactobacillus paracasei JCM1171- LC065035.1*	PTTC 1822	KY462713.1
DL131b	1,399 bp	100%	*Lactobacillus paracasei.toleranceJCM1171-LC065035.1*	PTTC 1827	KY462712.1
DL132	1,461 bp	99%	*Lactobacillus paracasei.paracaseiJCM8130-AP012541.1*	PTTC 1830	KJ508202.1
DL140	1,393 bp	99%	*Lactobacillus paracasei.toleranceJCM1171-LC065035.1*	PTTC 1845	MG523271.1
DL130	1,395 bp	99%	*Lactobacillus paracasei.toleranceJCM1171-LC065035.1*	PTTC 1846	MG523270.1

The evolutionary distances are in the units of the number of base differences per site. The constructed tree showed the evolutionary relationships of selected Lactobacillus isolates (Fig 1) with the division of Lactobacillus into three distant groups: DL132 and L. crustorum 90 in the first group (A), the second group (B) including L. fermentum 121 and DL131b, and the third group (C) including DL140 and DL130. An additional branch with DL200 (PTCC 1822) is located behind the A group. Despite the low production of lactic acid, DL200 has significant antimicrobial activity against Salmonella typhimurium, Escherichia coli, Bacillus subtilis, and Clostridium perfringens. It has antimicrobial activity against indicators even in neutralized supernatants. Therefore, the purification and characterization of the substances, which were responsible for the antimicrobial activity, are in progress.

4. Conclusions

The potential of selected isolates for use as food preservatives were studied and compared to previous reports in an effort to prevent the growth of food-borne pathogens. Our findings revealed that certain wild Lactobacilli could be considered as a potential alternative to chemicals for the purpose of controlling pathogenic bacteria to provide healthy food.

Moreover, the selected isolates use galactose as a carbon source when they are resident in the large intestine and can consume lactose thereby preventing D-lactic acidosis in the host.

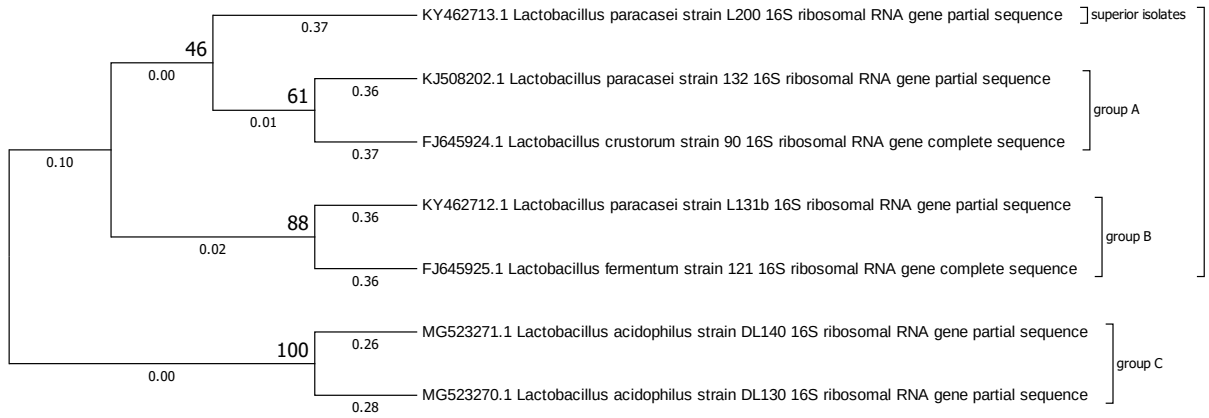

Figure 1. Neighbor-joining method (Saitou and Nei, 1987) was applied to construct phylogenetic tree of *Lactobacillus* isolate based on 16S rDNA sequence. Numbers above branches indicate percentage of trees in which the associated taxa clustered together with 100 replicates. *Lactobacillus acidophilus* strains are the related bacterial species as outgroups.

Conflict of Interest

The authors declares that they have no conflict of interest.

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Ethical approval

This article does/does not contain any studies with human participants or animals performed by any of the authors. This article does/does not contain any studies with human participants or animals performed by any of the authors.

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